REMARKS

I. Status of the Claims and Preliminary Remarks

The Applicant would like to thank the Examiner and his Supervisor, Bruce Campell, for the time taken on January 5, 2007 to discuss outstanding rejections in this application.

The Applicant would also like to thank the Examiner for the telephone call on November 1, 2006, during which the Examiner clarified that the statement in the Office Action at page 10, last full paragraph, should read as follows:

> The additional teachings of Bazan also indicate that those in the art would have been motivated to insert lysines for PEG attachment outside of the helices.

Claims 62, 66, 75, 76, 79, and 81-83 are pending in the instant application. Claims 75 and 76 have been amended to more particularly recite the subject matter Applicant regards as the invention. The amendments to the claims do not add new matter.

II. The rejection of claims 62, 66 and 81-83 under 35 USC §112, second paragraph, may be withdrawn.

At page 2 of the Office Action, the Examiner rejected claims 62, 66 and 81-83 under 35 USC §112, second paragraph, for assertedly being indefinite. Specifically, the Examiner asserted that it is not clear which residues form the referenced external loops and further asserted that the application gives conflicting information as to the residues that form the loops between the helices, and thus the claims are indefinite. Applicant respectfully traverses.

As a matter for clarification, Applicant points out that the Examiner, at page 3 of the Office Action, first full paragraph, incorrectly asserted that "Page 68 further identifies the AB helix as being residues 58-72 of the protein" when in fact the referenced disclosure at page 68 identifies the AB loop as being residues 58-72 r-hu-met G-CSF.

Turning then to the rejection, the Applicant submits that for any protein, precise designation of helix residues, i.e., identification of some specific and single residue (or junction of residues) at which, for example, a helix structure ends and a loop structure begins, is based on subjective analysis. This fact was known in the art as early as 1992 as evidenced by Zink, et al., (1992) FEBS Letters 314:435-439 (hereinafter "Zink" and attached hereto as Exhibit A). Specifically addressing the secondary structure of G-CSF as determined by nuclear magnetic resonance (NMR), Zink states at page 437:

> The precise definition of the start and end of helices depends on the criteria used and has to await detailed structure calculation. Taking into account that the length of a helix may vary by one or two residues, the four helices are defined as follows: 11-41, 71-95, 102-124, 144-170.

Thus, it was understood in the art that identifying a terminal helix residue could vary[1].

The Applicant notes that Zink's designation of G-CSF helix residues differs from the disclosure in the instant application[2] , but that Zink's structural analysis based on NMR fails to provide the detail that x-ray crystallography as provided in the instant application permits. Even with the more precise detail provided through x-ray crystallography, it must be kept in mind that atomic coordinates determined by x-ray crystallography represent a snapshot of a protein's structure. However, proteins in a biological system are not crystalline (i.e., static), and instead exist in an aqueous environment in which the structure is dynamic. Thus, while structural fluidity in the aqueous environment may not effect definitive assignment of certain amino acid residues as being part of a helix (or any secondary structure), precise identification of a single residue that is "the" terminus of any secondary structure is, as Zink discloses, still somewhat subjective. As such, variation in assignment of exact terminal helix residues in the instant application is both proper and necessary as one of skill in the art would appreciate from the disclosure of, e.g., Zink..

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[1] The Applicant notes that even with knowledge of Zink's amino acid helix designations, US patent application Publication No. 2006/0286068 (Exhibit B hereto) defines the four helices in Paragraph 40 as amino acids at positions 11-41 (helix A), 71-95 (helix B), 102-125 (helix C), and 145-170 (helix D). The differences in the publication compared to Zink are consistent with Zink's statement above.

[2] The Applicant notes that Zink et al., provides secondary structure information while the instant application provides the first disclosure of the 3-D structure of G-CSF at the atomic level (i.e., tertiary structure information).

Despite any asserted ambiguity, Applicant stresses that one of the central scientific contribution to the art in the instant application is the first disclosure of the three dimensional structure of G-CSF at the atomic level. With the information provided in Figure 5 and any of a number of computer programs known and available at the time the instant application was filed, a person of ordinary skill in the art would have been able to visualize the G-CSF structural snapshot and identify which residues are located at or near the termini of each helix. Thus, it is the information provided in Figure 5 that the skilled worker would look to, against the backdrop of the disclosure throughout the specification, to determine which modifications to the G-CSF structure are within the scope of the claims.

In view of the above, the rejection of claims 62, 66 and 81-83 under 35 USC §112, second paragraph, may be withdrawn.

III. The rejection of claims 75, 76, and 81-83 under 35 USC §102(b) or, in the alternative, under 35 USC §103(a), may be withdrawn.

At page 6 of the Office Action, the Examiner rejected claims 75, 76, and 81-83 under 35 USC §102(b) or, in the alternative, under 35 USC §103(a) as obvious over the disclosure of Shaw. Specifically, the Examiner asserted that Shaw teaches substitutions at positions corresponding to amino acid residues 17, 24, 35, 41, 147,148, 167 and 170, according to the Table at columns 13 and 14. Further, the Examiner asserted that amino residues 147, 148, 167 and 170 fall within helix D, and that none of these residues are reported as essential for structural integrity in the instant application. With respect to pegylation, the Examiner asserted that Shaw teaches G-CSF mutants that have been modified via pegylation at the each reactive lysine residue, and that such modification results in an increase in the in vivo serum half-life. Applicant respectfully traverses the rejection.

Applicant points out that claims 75 and 76 have been amended to remove recitation of "helix D" as a site for modification. As a result, parts b) of claims 75 and 76 only recite modification in either helix C or helix A and helix C, respectively. As the Examiner correctly points out, Shaw only teaches substitutions within helix A and helix D and nothing in Shaw discloses or suggests substitutions in helix C. Accordingly, the amendment to claims 75, 76, and 81-83 obviates the rejection under 35 USC §102(b) or, in

the alternative, under 35 USC §103(a) because the cited art fails to teach all of the limitations of the claims.

IV. The rejection of claims 62, 66 and 81-83 under 35 USC §103(a), may be withdrawn.

At page 8 of the Office Action, the Examiner rejected claims 62, 66 and 81-83 under 35 USC §103(a) as being directed to subject matter assertedly rendered obvious in view of the disclosure of Shaw, and further in view of the disclosure of Bazan et al. Specifically, the Examiner asserted that Shaw teaches substituting lysines at positions 17 and 41, and modifying at least one residue in helices A or D. With respect to Bazan et al., the Examiner asserted that the reference teaches that the loop regions of G-CSF would be appropriate for lysine insertion and pegylation as they were not expected to participate in G-CSF biological activity. During the above mentioned discussion on January 5, 2007, the Examiner clarified this position, indicating that Bazan and Shaw both demonstrate making G-CSF analogs with mutations in the helix regions but neither reference expressly teaches or demonstrates producing a G-CSF analog with a mutation in any loop region. The Examiner maintained that the worker of ordinary skill in the art, with the disclosure of Bazan teaching helix participation in receptor binding as a backdrop, would realize that substitutions could be made in the G-CSF loop regions to allow for PEG attachment and thus improve circulatory half-life of the protein. Applicant respectfully traverses.

MPEP §706.02(j) states:

> To establish a prima facie case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art and not based on applicant's disclosure. In re Vaeck, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). See MPEP § 2143 - § 2143.03 for decisions pertinent to each of these criteria. (emphasis added)

> The initial burden is on the examiner to provide some suggestion of the desirability of doing what the inventor has done. "To support the conclusion that the claimed invention is directed to obvious subject matter, either the references must expressly or impliedly suggest the claimed invention or the examiner must present a convincing line of reasoning as to why the artisan would have found the claimed invention to have been obvious in light of the teachings of the references." Ex parte Clapp, 227 USPQ 972, 973 (Bd. Pat. App. & Inter. 1985). See MPEP § 2144 - § 2144.09 for examples of reasoning supporting obviousness rejections.

The Applicant submits that the Examiner's reliance on Bazan, et al., is inappropriate because the reference neither expressly nor impliedly suggests limitations in the claims the Examiner purports.

Bazan, et al., do not rule out a functional significance for loop regions in cytokines having a four-helix structure. For example, at p. 352 in the sentence bridging the first and second columns, Bazan, et al., discuss receptor binding domains in cytokines of this type and notes,

> A strong clue to the existence of a receptor-binding structure code is revealed by an elegant set of mutagenic experiments by Cunningham and Wells that focus attention on an extended receptor-binding epitope in GRH formed by the exposed surface of helix D (with some adjoining residues from the nearby C-D loop and helix A; Fig. 2(b)).

The disclosure does not indicate the extent to which this work demonstrated or suggested participation of loop amino acid residues in receptor binding; "some adjoining residues" may suggest that not all of the C-D loop residues are involved but it does not rule out involvement of all C-D loop residues. This passage does, however, clearly implicate GRH loop residues in receptor binding, and whether this observation can be extended to all four-helix cytokines, and to G-CSF in particular, cannot be determined from Bazan, et al.

When addressing receptor binding in general, Bazan, et al., suggest the following.

> From this discussion a model of receptor-cytokine interaction emerges comprising a generic receptor structure that is able to dock helical cytokines that display a special recognition helix.

If the observations relating to GRH fit into this model, then the worker of ordinary skill must presumably first identify the "recognition helix" and then determine whether at least some loop residues adjacent the "recognition helix" are involved in receptor docking. The full disclosure of Bazan, et al., therefore cannot be extended to G-CSF binding (much less all four-helix cytokines) because the instant application is the first to demonstrate which domains in G-CSF are required for receptor binding.

For example, beginning at page 69, line 19, in the present application, G-CSF receptor binding is described as follows:

> The domains required for G-CSF receptor binding were also determined based on the above analogs prepared and the G-CSF structure. The G-CSF receptor binding domain is located at residues (with methionine being position 1) 11-57 (between the A and AB helix) and 100-118 (between the B and C helices). One may also prepare abbreviated molecules capable of binding to a G-CSF receptor and initiate signal transduction for selectively stimulating neutrophils by changing the external loop structure and having the receptor binding domains remain intact.
>
> Residues essential for biological activity and presumably G-CSF receptor binding or signal transduction have been identified. Two distinct sites are located on two different regions of the secondary structure. What is here called "Site A" is located on a helix which is constrained by salt bridge contacts between two other members of the helical bundle. The second site, "Site B" is located on a relatively more flexible helix, AB. The AB helix is potentially more sensitive to local pH changes because of the type and position of the residues at the carboxy and amino termini. The functional importance of this flexible helix may be important in a conformationally induced fit when binding to the G-CSF receptor. Additionally, the extended portion of the D helix is also indicated to be a G-CSF receptor binding domain, as ascertained by direct mutational and indirect comparative protein structure analysis. Deletion of the carboxy terminal end of r-hu-met-G-CSF reduces activity as it does for hGH, see, Cunningham et al. Science 244: 1081-1084 (1989). Cytokines which have similar

> structures, such as IL-6 and GM-CSF with predicted similar topology also center their biological activity along the carboxy end of the D helix, see Bazan Immunology Today 11: 350-354 (1990).

The instant specification demonstrates the importance of these domain with a multitude of variants described throughout the examples.

From the above, it is important to realize that the binding domains found in G-CSF are not identical to those Bazan, et al., describe for GRH, and without the G-CSF binding domain information made available in the instant application (which is undeniably absent from the disclosure of Bazan, et al.), the worker of ordinary skill cannot predict involvement, or lack thereof, of any G-CSF loop amino acid residue in receptor binding from the Bazan, et al., disclosure.

If Bazan, et al., suggest modification of any cytokine amino acid residues, the disclosure is limited to making changes in helix residues and not loop residues. For example, at page 353, left column, Bazan et al, describe implications for protein design:

> Most of the protein fold of a helical cytokine would serve an essential function as a structural scaffold for presenting the recognition helix to the receptor. Bioactivity of the cytokine may be maintained by judicious alteration of the structure that does not disturb the receptor-binding epitope. For example, Landgraft et al have engineered a more stable, semi-synthetic version of IL-2 by incrementing the amphiphilicity of one or more of the core helices of the 4-α helix bundle. Conceivably, this approach could extend to the wholesale grafting of a receptor-binding helix to an engineered, host protein fold. At the other extreme, 'minimal' cytokines that retain a fair level of bioactivity could be equivalent to an amphiphilic helical peptide. (This is a functional strategy that is apparently employed by a class of small hormone-like proteins.) Bioactive helices that are stable in solution could be linked to form higher aggregates that exist as 2-α helix hairpins, 2-α/2-α or 4-α bundles, novel molecules that may be particularly effective in generating cytokine-neutralizing antibodies.
>
> The (convergent) similarity of certain key cytokine residues within the aligned recognition helices of Fig. 3(b) suggests a parallel similarity of binding sites in the set of cognate receptors. Progressive replacement of the helix residues (for example in the conversion of PRL to GRH-like cytokine) may probe individual amino acid contributions to specific binding. (Emphasis added.)

Thus, Bazan et al., disclose possible implications of modifying helical regions, and suggests the creation of proteins having improved biological properties through modification(s) in one or more helix regions by pointing to the results of prior work by Landgraft et al.

The Examiner's contention that Bazan et al., indicate "the loop regions would be appropriate regions for lysine insertion and PEG attachment as they were not expected to participate in the protein's activity" is conjecture that is not supported in Bazan et al.[3] To the contrary, while Bazan, et al., does not definitely show which, if any, loop residues are required for receptor binding in at least GRH, the disclosure does suggest that GRH loop residues are involved. Were loop modifications in four-helix cytokines as insignificant as the Examiner suggests, to structure and/or function in any of the known cytokines having this structure, certainly one would expect that there would be art describing such an analog for at least one of the multitude of other similarly structured cytokines well known in the art (*see, e.g.,* Figure 2 in the present application) that would evidence the Examiner's assertion and support the rejection. No such art, however, has been cited, and absent such support, the Applicant submits that the rejection of claims 62, 66 and 81-83 under 35 USC §103(a) must be withdrawn..

V. The rejection of claims 62, 66, 75, 76, 79 and 81-83 under 35 USC §103(a), may be withdrawn.

At page 10 of the Office Action, the Examiner rejected claims 62, 66, 75, 76, 79 and 81-83 under 35 USC §103(a) as being obvious over Shaw, in view of Bazan et al., and further in view of Bowie et al. Specifically, the Examiner asserted that in addition to the teachings of Shaw and Bazan (described briefly above), Bowie et al., teach that conservative mutations (e.g., in G-CSF) would generally expected to be operable. Applicant respectfully traverses.

[3] The Applicant notes that in prosecution of US patent application Serial No. 10/318,966 (Nissen et al.,) (hereinafter the '966 application) which claims similar subject matter as the present application and is being examined by the same Examiner as in the instant application, a rejection under 35 USC §103 was also made which relied on all of the references cited in the instant rejection. However, in the '966 application, the Examiner further relied on US Patent 5,790,420, in part, for the specific disclosure suggesting the loop regions as targets for attachment of PEG molecules.

The Applicant repeats the previous arguments set forth above with respect to Shaw and Bazan et al. Briefly, Shaw fails to teach modifying G-CSF by substituting residues in loop regions, and Bazan et al., fails to motivate the skilled worker to make changes in loop regions. Bowie et al., does not rectify this deficiency. Indeed, Bowie et al., is completely silent with regard to G-CSF, let alone identifying any specific regions in G-CSF and making modifications therein. As a result, the combined disclosures of Bowie et al, Shaw and Bazan et al., cannot render obvious subject matter of claims 62, 66, 75, 76, 79 and 81-83 and the rejection under 35 USC §103(a) must be withdrawn.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to pass this application to issue.

Dated: March 19, 2007

Respectfully submitted,

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Volume 314, number 3, 435–439 FEBS 11908 December 1992

Secondary structure of human granulocyte colony-stimulating factor derived from NMR spectroscopy

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Received 6 November 1992

Recombinant ^{15}N-, ^{13}C-labeled human granulocyte colony-stimulating factor (rh-metG-CSF) has been studied by 2D and 3D NMR using uniformly labeled protein as well as residue-specific ^{15}N-labeled samples. Assignment of the ^{1}H, ^{15}N backbone, and 60% ^{1}H sidechain resonances has enabled the determination of the secondary structure of the protein. The secondary structure is dominated by α-helical regions with four stretches of helices between residues 11–41, 71–95, 102–124 and 144–170.

Nuclear magnetic resonance; Secondary structure; Human granulocyte colony-stimulating factor

1. INTRODUCTION

Human granulocyte colony-stimulating factor (h-G-CSF), a 174-residue glycoprotein (MW = 19 kDa, pI = 6.1), is one of the hematopoietic growth factors that play a major role in the process of blood formation [1]. The protein is produced in monocytes and fibroblasts. It serves three purposes: it causes the proliferation of hematopoietic precursor cells, induces the differentiation of the precursor cells into mature neutrophilic granulocytes, and activates neutrophilic granulocytes. The sugar chain, linked to Thr-133-^{17}O, is not required for biological activity [2,3]. Circular dichroism measurements showed that h-G-CSF is largely α-helical with little or no β-structure [4]. Here we describe the secondary structure of rh-metG-CSF, a recombinant h-G-CSF which differs from the native protein at the N-terminal residue (methionine). Sequential assignment with conventional 2D NMR techniques [5] could not be carried out due to severe overlap of resonances in the 2D spectra for a protein of this size. The small variation of the chemical shifts of the α- and NH-protons, a consequence of the predominantly α-helical structure, further complicated the assignment. The sequential assignment of the backbone proton and nitrogen resonances was accomplished with heteronuclear 2D and 3D spectra for both the uniformly ^{15}N-labeled and the residue specific ^{15}N-labeled samples, mostly from the 3D NOESY-HMQC spectra [6–8]. The equivalent TOCSY spectra provided only limited information as most of the expected signals were weak or missing due to short proton T_2-relaxation times for rh-metG-CSF. The linewidths of the proton resonances were typically 30 Hz; slightly but noticeably larger than the expected linewidths for a protein of this size. We also recorded the HNCA triple resonance experiment [9] for a uniformly ^{15}N-,^{13}C-labeled sample. This experiment enabled identification of residues not located in α-helices.

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2. MATERIALS AND METHODS

The rh-metG-CSF was expressed in the cytoplasm of *E. coli* as insoluble inclusion bodies. The correctly folded protein was extracted from the inclusion bodies by renaturation of the unfolded protein in a 0.8 M arginine buffer after solubilization of the inclusion bodies in 6 M guanidinium chloride [10,11]. The final step of the preparation involved conventional column chromatography.

For the uniformly ^{15}N-labeled sample, rh-metG-CSF was expressed in the *E. coli* strain TG 1 in a minimal medium [12]. The medium was slightly modified by substituting $(NH_4)_2HPO_4$ for [^{15}N]ammonium chloride (at a concentration of 1 g/l) as the sole source of nitrogen. The purified protein was dialyzed against sodium phosphate (4 mM, pH 4.0) and lyophilized. The samples were typically 1–2 mM in protein and 50–60 mM in sodium phosphate, after addition of 0.5 ml H_2O/D_2O (9:1) or 0.5 ml D_2O to the lyophilized powder, and after adjustment of the pH to 3.5. This low pH value was necessary due to the low solubility of rh-metG-CSF at higher pH values. For the residue specific ^{15}N labeling, the *E. coli* strain C600+ was used in a minimal medium enriched with all 20 amino acids [13]. The ^{15}N-labeled amino acid was added at concentrations between 0.25 and 1.0 g/l, half of which was added to the medium at the beginning of fermentation, the other half on induction. The rh-metG-CSF was labeled with [^{15}N]glycine, [^{15}N]leucine, [^{15}N]valine, [^{15}N]alanine and [^{15}N]lysine. Cross-labeling of serine was achieved by a deliberate omission of serine from the amino acid-enriched medium during labeling with [^{15}N]glycine. The uniformly ^{15}N-,^{13}C-labeled sample was expressed in a minimal medium containing 1 g/l [$^{13}C_6$]glucose as the sole carbon source [12]. All labeled preparations were purified by the procedure outlined above. Table 1 summarizes the various labeled samples prepared and the cross labeling observed [14].

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The NMR spectra were recorded on a Bruker AMX600 spectrometer operating at 600.14 MHz for protons. For all experiments the temperature was set to 27°C. Spectra were acquired and processed in the phase sensitive mode using time proportional phase incrementation (TPPI) [15] in all dimensions. Suppression of the water signal was achieved by presaturation or jump–return pulses [16–18]. The mixing time employed for the 3D NOESY-HMQC [6] was set to 120 ms; the MLEV-17 mixing sequence used for the 3D TOCSY-HMQC [6] spectra was set to 40 ms and 70 ms. 2D spectra were processed with UXNMR; the 3D spectra were processed with our in-house software (GC-NMR).

3. RESULTS AND DISCUSSION

The predicted α-helical structure for G-CSF has been confirmed by 2D NOESY spectrum. There are about 200 NH–NH cross peaks (on both sides of the diagonal) in the spectrum. The NH–NH connectivities are characteristic of the α-helical structure [5,19]. Further indication of the helical structure is provided by chemical shift ranges of α-protons (Fig. 1). From the total of 176 $C^{\alpha}H$ resonances that are expected to be present in the spectrum, only five appeared below 4.7 ppm (the chemical shift of water). This upfield shift of 0.2–0.6 ppm relative to random coil values is characteristic of helical structures [20]. In the ^{1}H–^{15}N 3D NOESY-HMQC spectra the 2D overlap was mostly resolved, although some degree of overlap still remained. Fig. 2 shows the 2D NOESY projection of the 3D NOESY-HMQC spectrum. In the 3D TOCSY-HMQC spectrum, the only visible magnetization transfers were from the NH- to the α-protons. The 3D TOCSY-HMQC with a mixing time of 70 ms showed more cross peaks than the same experiment using 40 ms, and also few peaks from the

Table 1

^{15}N labeling of rh-metG-CSF by amino acid type

^{15}N-labeled amino acid in the medium	Cross-labeling observed
[^{15}N]Ala	–
[^{15}N]Gly[a]	Ser
[^{15}N]Gly[b]	Ser
[^{15}N]Leu	–
[^{15}N]Val,[α-^{15}N]Lys	Ala

[a] [^{14}N]Ser added to the medium; low level of cross-labeling.
[b] [^{14}N]Ser omitted from the medium; deliberate high level of cross-labeling.

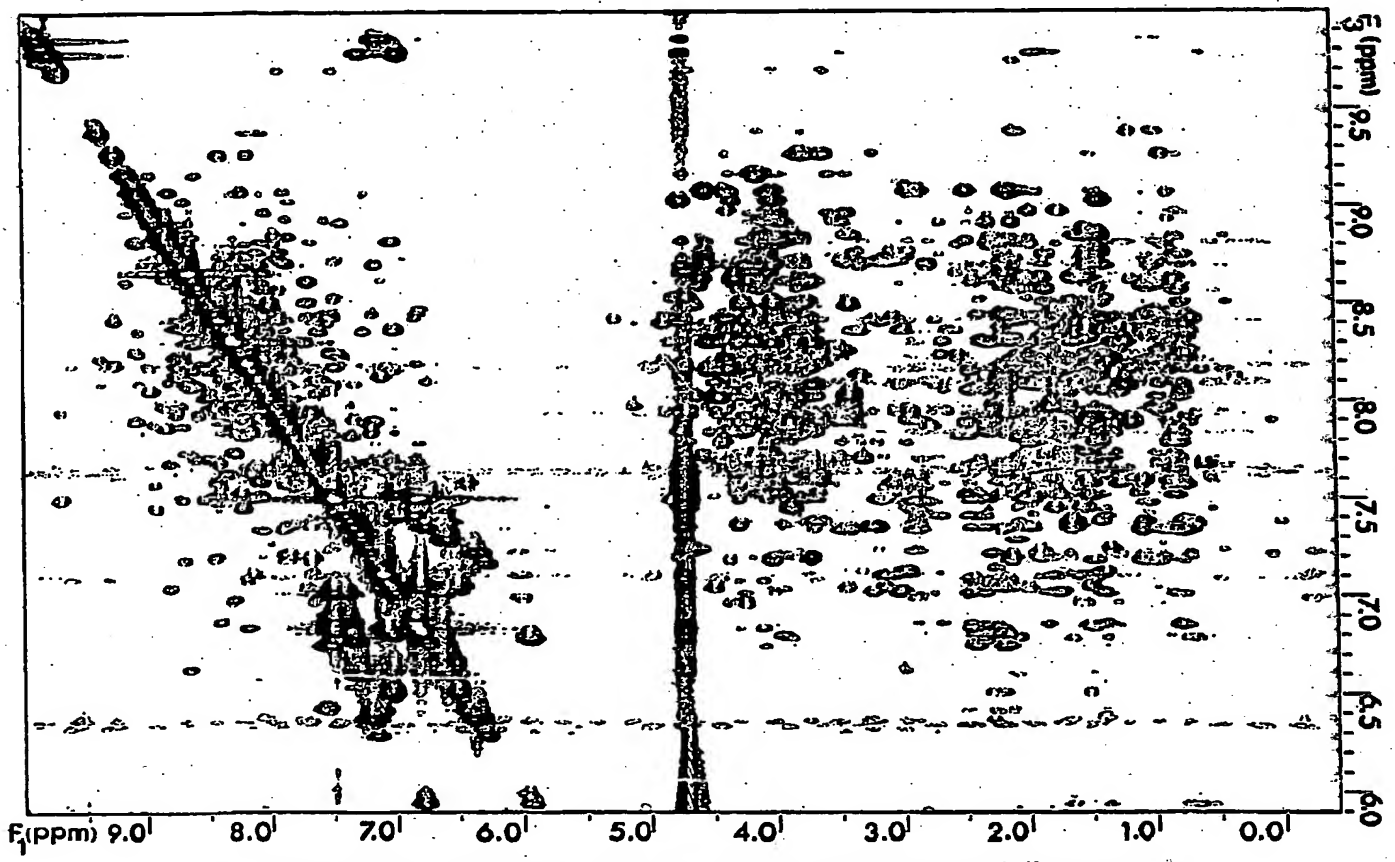

Fig. 1. The 2D-NOESY projection of the 600 MHz 3D NOESY-HMQC spectrum of [^{15}N]rh-metG-CSF.

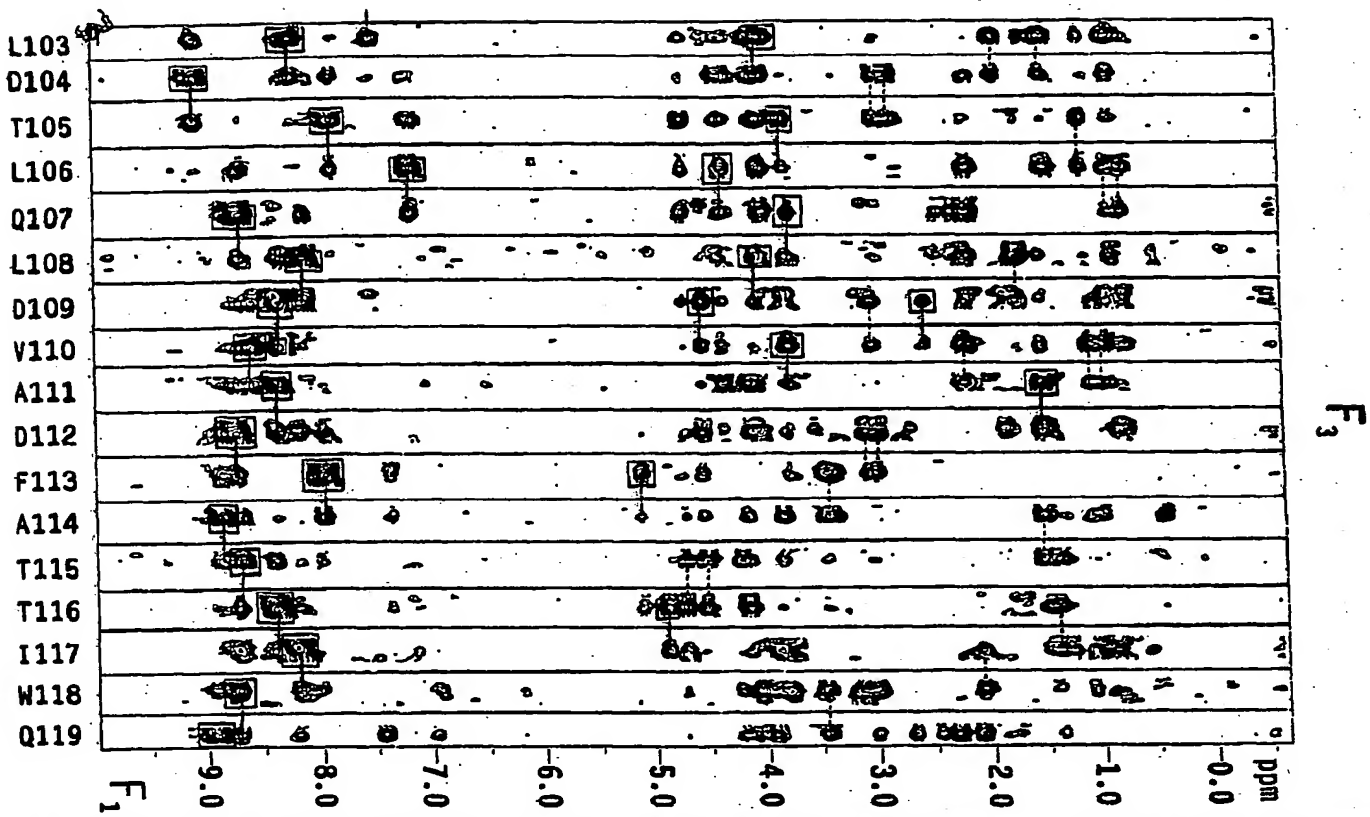

Fig. 2. Strips from the 3D NOESY-HMQC spectrum of [^{15}N]rh-metG-CSF showing resonances of residues L103-Q119 belonging to an α-helix. The strips, 8–11 points (50–66 Hz) wide, are taken from the 3D NOESY-HMQC at the ^{15}N- and ^{15}NH-chemical shift of the residues of interest. Rectangles mark intraresidual peaks identified from the 3D TOCSY-HMQC. Solid lines indicate sequential NH(i)–NH(i−1), NH(i)–C$^{\alpha}$H(i−1), NH(i)–C$^{\beta}$H(i−1) and NH(i)–C$^{\gamma}$(i−1) connectivities. Dashed lines are drawn when the peaks from residue (i−1) could not be identified as intraresidual in the 3D TOCSY-HMQC.

NHs to β-protons. Altogether 105 signals could be identified as intraresidual α-protons, while only 38 β and γ resonances were seen. Therefore the majority of the spin systems were identified with residue specific ^{15}N labeling.

The sequential assignment was started with the NH–NH connectivities. Most of the residues showed strong NH(i)–NH(i−1) and NH(i)–NH(i+1) cross peaks. The directionality of the sequence could often be determined by a C$^{\alpha}$H(i)–NH(i+1) cross peak between the residues involved in the NH(i)–NH(i+1) connectivity. Also, C$^{\beta}$H–NH(i+1) peaks could sometimes be identified and served as further proof for the sequential assignment. The C$^{\alpha}$H–NH(i+3) cross peaks were identified in 90% of residues that are in the helical conformation. By this procedure, protein segments varying in length from three to nine residues were found. All these segments contained the residue specific ^{15}N-labeled amino acids. Fig. 2 shows an example of the sequential assignment in the 3D NOESY-HMQC spectrum for residues Leu-103 to Gln-119. In Fig. 3, a schematic representation of the sequential and medium-range NOEs is displayed. It is clear from the pattern of short- and medium-range NOEs that the secondary structure of rh-metG-CSF in solution is dominated by four helices. The precise definition of the start and the end of helices depends upon the criteria used and has to await detailed structure calculations. Taking into account that the length of a helix may vary by one or two residues, the four helices are defined as follows: 11–41, 71–95, 102–124 and 144–172.

Turns, which are also characterized by NH–NH NOEs, can be distinguished from helices by the chemical shift of the α-protons and by characteristic C$^{\alpha}$H(i)–NH(j) connectivities [19]. The chemical shifts of α-protons in turns are similar to random coil values, while in helices an upfield shift is observed. The NH–NH, C$^{\alpha}$H(i)–NH(j) connectivities, and characteristic random coil chemical shifts are observed for residues 66–70, 129–131, 133–137 and 139–143. Hence these residues are likely to be in turns rather than in other non-helical structures. In the ^{15}NH–^{13}C-planes of the 3D triple resonance HNCA experiment [9] two signals are expected

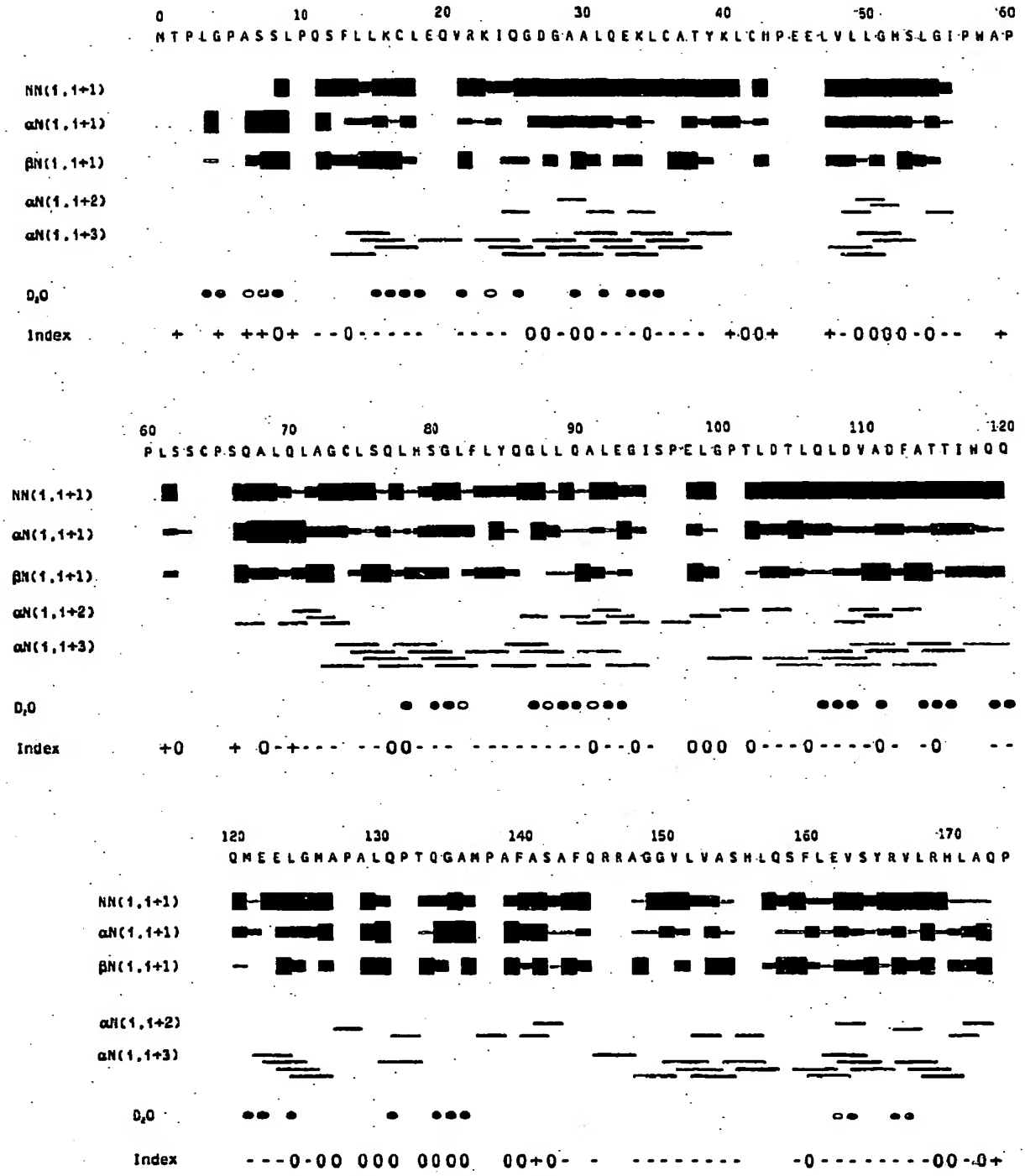

Fig. 3. Summary of the short-range NOEs. The intensities of the NOEs are reflected in the thickness of the bars (except for the $d_{\alpha N}(i,i+2,3)$ connectivities). The $C^{\alpha}H(i)$–$C^{\alpha}H(i-1)$(Pro) NOE is shown along the same line as the $C^{\alpha}H(i)$–NH(i+1) connectivities. Filled bars indicate the data that could be semiquantified, open bars indicate the data that could not be semiquantified due to spectral overlap. Circles indicate residues that show slow NH-exchange and were therefore still seen in the spectra in D_2O after 2 days. In cases where two residues have both identical ^{15}NH- and ^{15}N-chemical shift circles are left open. The bottom row shows an index that is a measure of the $C^{\alpha}H$-shift relative to random coil values, + and − indicating a shift to higher and lower ppm values, respectively. The 0 is given where no or only a small shift was observed.

for a residue in a non-helical conformation: the intraresidual connectivity NH(i)–C^{α}(i) and the interresidual connectivity NH(i)–C^{α}(i−1). Due to the larger heteronuclear one-bond coupling constant ($^1J_{CN}$ = 11 Hz) the intensity of the former signal is expected to be stronger than that of the latter ($^2J_{CN}$ = 7 Hz). This was indeed observed for residues in fragments 3–4 and 6–9. The same pattern was also observed for residues 66–69, 129–130, 133–137 and 139–143 which were already identified as being in turns from the 3D NOESY-HMQC data. In contrast to the predicted coupling patterns of the non-helical amino acids, residues located in helices should exhibit no or only a weak intraresidual NH(i)–C^{α}H(i) peak. This is due to the decreased coupling constants $^1J_{CN}$ and $^2J_{CN}$ which results in a decreased magnetization transfer from the amide proton to the alpha carbons. Indeed, only few NH(i)–C^{α}H(i) peaks were observed for the helical residues in G-CSF.

In this paper we have described the secondary structure of rh-metG-CSF. The highlight of the G-CSF structure is the presence of four helices between residues 11–41, 71–95, 102–124 and 144–170. Turns are located at residues 66–70, 129–131, 133–137 and 139–143. Calculations of the 3D structure will give a more detailed picture of the 3D solution structure of rh-metG-CSF.

Acknowledgements: This work was supported by a research grant from the Bundesministerium für Forschung und Technologie.

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